

23 AUG 2001

- 1 -

FLAVOR
~~FLAVOUR~~/AROMA MATERIALS AND THEIR PREPARATION

41
32
Background

The present invention relates to flavour/aroma materials and the preparation of such materials and key intermediates.

5 It is particularly (though not exclusively) concerned with vanilla flavour materials and related materials.

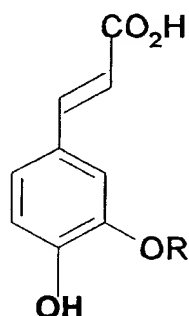
Supplies of natural vanilla bean extracts suffer from shortages of supply and variability in quality. Despite a wide range of natural flavour chemicals having become
10 commercially available over the last 10-15 years, no entirely satisfactory natural vanillin flavour chemical product or vanilla flavour has yet been developed. The main
difficulties in developing a cost-effective vanillin product are firstly the unavailability of the preferred raw material, ferulic acid; and secondly the difficulty in finding
15 microbial strains that can accumulate vanillin due to its ease of further metabolism, for instance to vanillic acid, and its inhibitory effect on the metabolism of cells. The main difficulty in developing a natural vanilla flavour is
20 the large number of different chemicals that together contribute to the superior flavour and aroma of vanilla bean extracts. In addition for the minor usage of vanilla in fragrances a colourless solid product is required rather than the coloured ethanol-water vanilla bean extracts.

25 Our earlier application WO-A-96/39859 discloses the production of some phenolic materials by the enzymatic hydrolysis of plant materials. Thus ferulic acid (1a) was produced by enzyme treatment of wheat germ or wheat bran.

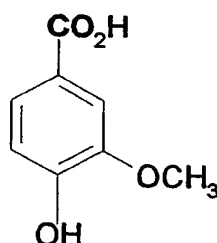
- 2 -

Caffeic acid (1b) was produced by enzyme treatment of sunflower meal. Ferulic acid and esters thereof are valuable as precursor compounds and also as ingredients of foods and cosmetics, e.g. serving as antioxidants.

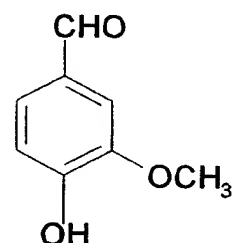
5

(1a)R-CH₃

(1b)R=H



(2)

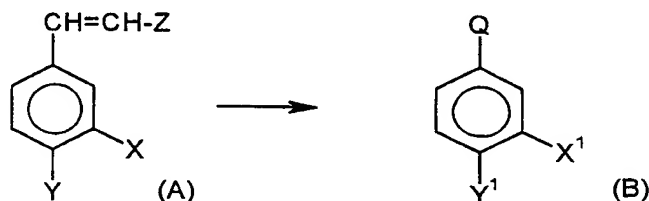


(3)

Various workers have reported the microbial conversion of ferulic acid (1a) into vanillin (3) either directly (e.g. DE-A-19532317) or via vanillic acid (2) (FR-A-2724394).

Outline of the Invention

The invention provides, inter alia, a method of converting a first composition comprising one or more species of formula (A) into a second composition comprising one or more species of formula (B):



where X, Y, X' and Y' are independently selected from H, OH and OMe; Z is CO₂H, CO₂carb (where carb represents a carbohydrate residue), CHO or CH₂OH, and Q is CHO, CO₂H or CH₂OH, said method comprising treating said first composition with one or more microorganisms under conditions such that

- 3 -

(A) is converted into (B); said microorganism(s) being selected, from (a) *Pseudomonas putida*; (b) *Rhodotorula* species and other yeasts capable of converted ferulic acid into vanillic acid; (c) microorganisms possessing both
5 ferulic acid esterase activity and intra-sidechain cleavage activity such that they are capable of converting ferulic acid glycosides into vanillin and/or vanillic acid; and (d) *Micromucor isabellinus* or *Aspergillus fumigatus* strains capable of converting vanillic acid into vanillin.

10 The process may include a preliminary step of obtaining said first composition comprising ferulic acid from a plant material by a process comprising:

(a) treating the plant material to produce a solution containing a ferulic acid ester; and

15 (b) treating said solution with an enzyme composition having ferulic acid esterase activity under conditions such that ferulic acid esters are converted into ferulic acid. N.B the esters generally involve carbohydrate residues, particularly sugar residues, and are sometimes termed
20 glycosides.

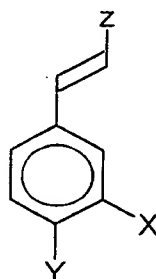
The invention further provides several microorganisms useful in methods of the invention. These include several deposited strains and mutants thereof (which may be produced by chemical or other conventional mutagenesis or by genetic
25 engineering). As well as intact organisms, use may be made of extracts and isolated enzymes.

Aspects of the present invention include the following.

- 4 -

A) A process for use in preparing a flavour/aroma composition containing a plurality of flavour/aroma components, said process having the step(s) of

(i) treating a plant material or plurality of plant materials to produce a precursor compound and preferably a plurality of precursor compounds (separately or mixed) preferably comprising two or more 1-phenylalkene species, preferably of formula (4):



where X and Y are independently selected from H, OH and OMe and Z is CO₂H, CO₂carb (where carb represents a carbohydrate residue) CHO or CH₂OH, most preferably comprising the compound (4) wherein X=OMe, Y=OH and Z=CO₂H (ferulic acid) and preferably also the compounds wherein X=H, Y=OH and Z=CO₂H (coumaric acid) and X=OH, Y=OH and Z=CO₂H (caffeic acid) ; and preferably

ii) subjecting said precursor compound or compounds (with or without separation from the plant material residues) to one or more biotransformations to produce a flavour/aroma composition. For precursor compounds having a benzene ring bearing a substituent -CH:CH-CO₂H (e.g. compounds of structure (4)), biotransformations may generate compounds in which this substituent has been converted into -CO₂H and/or -CH₂OH and/or -CHO.

- 5 -

The term "plant material" as used herein includes material such as the meal or pulp produced by mechanical processing of plants, and the residues left after extraction of seed oils etc..

5 Particularly preferred plant materials for step (i) include maize, wheat, rice, sugar beet and parts thereof, particularly waste materials from their normal uses e.g. rice bran and cereal fibres. For example maize fibre and wheat fibre may be derived from dry or wet milling. Sugar beet fibre may be derived from pulp. A mixture of plant materials may be employed to give a desired mixture of precursors.

Step (i) may involve:

(a) treatment of plant material (damp or dried) with acid (preferably citric acid, e.g. provided by addition of lemon or lime juice) to release glycosides of at least ferulic acid, generally with heating e.g. to 50-250°; and

(b) treatment of the glycoside-containing mixture with base (e.g. an alkali metal bicarbonate) or one or more enzymes with ferulic acid esterase ("FAE") activity
20 to release ferulic acid. Suitable enzymes include Hemicellulase (from Amano, derived from Aspergillus spp.) and/or Celluzyme (Novo Nordisk) and/or enzymes from Hemicola insolens, available as Pentopan, Biofeed Plus or Biofeed Beta (Novo Nordisk). These enzymes have some xylanase activity,
25 in addition to their FAE activity. If required, additional sources of xylanase can be added to supplement the xylanase activity already present. (Note: the so-called glycosides of

- 6 -

ferulic acid etc. are in fact esters of carbohydrate residues rather than conventional glycoside ethers).

In step (a), citric acid is suitable because it is reasonably strong, heat stable, cheap, active on a range of ferulic acid containing materials, non-volatile, and not inclined to cause side reactions. It is adequately soluble in the cereal "mashes". It can easily be recovered as an insoluble salt (e.g. calcium), for reuse. It is a "natural" material, which is approved for food use. Alternatives include other organic polycarboxylic acids, particularly hydroxyacids, such as isocitric, tartaric, malic, fumaric and succinic acids.

Solutions containing suitable acids may be used, e.g. a grape-derived solution containing tartaric acid; or a fermentation medium containing citric or malic acid.

A microorganism may provide the activities required to carry out step (i) (b) and step (ii) (biotransformation of ferulic acid and/or other precursor compounds). For example we have developed strains of Aspergillus niger, A.flavus, and Penicillium chrysogenum having both the necessary FAE and alkene cleavage activities for acting on the product of the step (a) to convert ferulic acid glycoside into vanillic acid.

Step (i) can also be effected by treatment of plant material (e.g. maize fibre) with aqueous alkali such as a hydroxide, carbonate or bicarbonate of an alkali metal or alkaline earth metal. Sodium bicarbonate is preferred.

- 7 -

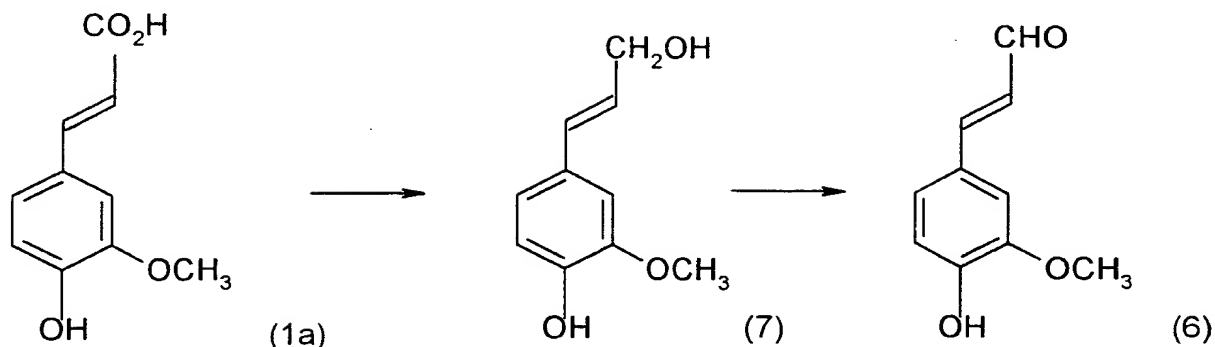
A product mixture containing vanillic acid and other materials (e.g. p-hydroxybenzoic acid from coumaric acid) may undergo one or more further biotransformations without isolation of individual components, e.g. converting vanillic acid into vanillin and effecting corresponding transformations of other components.

B) Preparation of vanillin comprising the biotransformation of vanillic acid (2) into vanillyl alcohol (5), and the biotransformation of vanillyl alcohol (5) into vanillin (3). Generally the two steps will employ different microorganisms. N.B compounds such as vanillyl alcohol may be valuable flavour chemicals in their own right.

The steps may be novel in their own right. The reduction of vanillic acid to vanillyl alcohol may be effected by Zygorhynchus moelleri.

C) Preparation of vanillin comprising the biotransformation of vanillic acid (2) directly into vanillin (3) by means of strains of microorganisms such as Aspergillus fumigatus or Micromucor isabellinus.

D) Preparation of vanillin comprising the biotransformation of ferulic acid into vanillin, e.g. by a strain of Pseudomonas putida.



E) Preparation and isolation of substances,

5 particularly substances prone to further reaction (e.g. aldehydes) or prone to inhibiting microorganisms (e.g. vanillin) by effecting a biotransformation using a microorganism in an aqueous phase which is brought into contact with a second phase into which products pass. This "in situ product removal" ("ISPR") may employ a vegetable oil as the second phase. Products may subsequently be recovered from the second phase, e.g. by crystallisation or solvent extraction. This process of ISPR can be employed in step (ii) of process A (above), or in processes B, C and D.

15 F) A vanilla flavour/aroma composition which is the product of a process according to (A) above or is a blend of one or more such products and/or one or more substances prepared by a process according to (B), (D), or (E), above and/or one or more vanilla flavour chemicals from other sources and/or a vanilla bean extract.

20 G) Use of Pseudomonas putida for converting ferulic acid into vanillic acid. The raw material is preferably the mixture obtained by treating plant material such as cereal

- 9 -

fibre with citrate and FAE enzyme (as described in section A above), without isolation of the ferulic acid.

H) Various transformations as described above may be applied to other substrates. This may lead to further useful flavour/odour components. For example benzoic acid, 4-hydroxybenzoic acid and 3,4-dihydroxybenzoic acids can be converted into the corresponding aldehydes and/or benzyl alcohols.

I) A method of isolating strains of microorganisms for use in processes of the invention. Material (e.g. soil samples) containing a multiplicity of strains is used to produce a multiplicity of colonies, e.g. on agar, and individual colonies are tested for useful activity by means of a reagent suitable for detecting aldehydes. For example, 2,4-dinitro-phenylhydrazine produces orange/red zones around aldehyde-producing colonies and a dark yellow zone around an alcohol producing colony.

J) Strains isolated by method I) and mutants thereof (obtained naturally, by conventional mutagenesis or by genetic engineering). This includes heterologous organisms which have been transformed so that they have derived activities of the "parent" organism from which the transforming nucleic acid was directly or indirectly derived.

Some particularly preferred strains have been deposited under the Budapest Treaty. Brief details follow.

a) Strains deposited with NCIMB (NCIMB Ltd, 23 St Machar Drive, Aberdeen, A24 3RY, GB)

- 10 -

1. Brevundimonas vesicularis (Zyl 295)-NCIMB 40987-Gram negative bacterium (deposited 18/11/98)

2. Pseudomonas putida (Zyl 503)-NCIMB 40988-Gram negative bacterium (18/11/98)

5 b) Strains deposited with IMI (CABI Bioscience UK Centre Egham, Genetic Resource Collection, Bakeham Lane, Egham, Surrey TW20 9TY, GB)

1. Rhodotorula glutinis (Zyl 717)-IMI 379896-Yeast, producing a red/orange pigment (20/11/98)

2. Aspergillus flavus (Zyl 714)-IMI 379895-Filamentous fungus producing light green spores (20/11/98)

3. Aspergillus fumigatus (Zyl 747)-IMI 379902-Filamentous fungus producing blue/grey spores (20/11/98)

4. Trichoderma koningii (Zyl 751)-IMA 379903-Filamentous fungus producing green spores (20/11/98)

5. Aspergillus niger (Zyl 759)-IMI 379904-Filamentous fungus producing brown/black spores (20/11/98)

6. Micromucor isabellinus (Zyl 849)-IMI 379893-Filamentous fungus producing pale brown spores (20/11/98)

7. Zygorhynchus moelleri (Zyl 851)-IMI 379899-Filamentous fungus, with aerial hyphae producing black spores (20/11/98)

8. Penicillium chrysogenum (Zyl 860)-IMI 379900-Filamentous fungus producing blue spores (20/11/98)

9. Pseudomonas putida (Zyl 581)-IMI 382568, Gram negative bacterium; deposited 31/1/00

- 11 -

The use of those strains is illustrated in the following examples. Of course other strains of the same species may be used to carry out the same transformations.

The invention will now be explained in more detail, with reference to some specific examples.

General Experimental Conditions

In the following examples, where organisms are grown in culture broth, the growth medium can contain specified amounts of either, or both, of a vitamins supplement and a trace elements supplement.

These were prepared as follows.

Vitamins supplement: biotin (2mg L^{-1}), folic acid (2mg L^{-1}), pyridoxine (10mg L^{-1}), riboflavin (5mg L^{-1}), thiamine (5mg L^{-1}), nicotinic acid (5mg L^{-1}), pantothenic acid (5mg L^{-1}), vitamin B12 (0.1mg L^{-1}), 4-aminobenzoic acid (5mg L^{-1}), and thioacetic acid (5mg L^{-1}).

Trace elements supplement: concentrated hydrochloric acid (51.3 mL L^{-1}), MgO (10.75g L^{-1}), CaCO_3 (2.0g L^{-1}), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (4.5g L^{-1}), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (1.44 g L^{-1}), $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (1.12 g L^{-1}), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.25g L^{-1}), $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ (0.28g L^{-1}), and H_3BO_3 (0.06 g L^{-1}).

Analysis of coniferyl alcohol, coniferaldehyde, caffeic acid, coumaric acid, ferulic acid, vanillic acid, vanillyl alcohol and vanillin was carried out using high performance liquid chromatography (hplc) using the following conditions:

Column	Spherisorb C_{18}
Mobile phase	80:20 deionised water:acetonitrile containing 1% acetic acid

- 12 -

Flow rate 1.75 mL min⁻¹
Detection Ultraviolet at 290nm

Analysis of 4-hydroxybenzoic acid and 4-hydroxybenzaldehyde was carried out using high performance
5 liquid chromatography (hplc) using the following conditions:

70/30
Column Spherisorb C₁₈
Mobile phase 80:20 deionised water:acetonitrile containing
1% acetic acid
Flow rate 2 mL min⁻¹
10 Detection Ultraviolet at 275nm

Alternatively, analysis of 4-hydroxybenzoic acid and 4-hydroxybenzaldehyde was carried out using thin layer
chromatography (tlc) using the following conditions: silica
plates eluting with petroleum ether (40-60): ethyl acetate
15 (50:50) and visualisation with UV or dinitrophenylhydrazine
solution (0.4% in 2M HCl).

A) Use of Plant Materials

Maize fibre is an example of a cheap raw material which
can provide raw materials for use in the preparation of
20 flavour/aroma materials. Treatment with alkali leads to the
liberation of ferulic acid, and lesser amounts of other
materials, notably coumaric acid. The mixture can be
subjected to biotransformation, e.g. to produce a mixture of
vanillic acid and 4-hydroxybenzoic acid, which could be
25 subjected to further biotransformations, e.g. converting the
carboxylic acid groups to -CHO and/or -CH₂OH.

Examples 1A: Ferulic and coumaric acids from maize

- 13 -

(a) Use of sodium hydroxide: to 500g of ground maize fibre was added 1 litre of 1M sodium hydroxide solution and the resulting suspension was thoroughly mixed then allowed to stand at ambient temperature (22°C) for 15 hours. Then 1 litre of ethyl acetate and 100mL of concentrated hydrochloric acid were added and the suspension mixed. The ethyl acetate phase was separated and the fibre suspension re-extracted with a further 1 litre of ethyl acetate. The combined organic solvent phases were dried (Na_2SO_4) and evaporated to dryness to yield a thick oil. Repeated washing of this oil with n-hexane gave a pale yellow solid comprising 33% ferulic acid and 2.9% coumaric acid.

(b) Use of sodium bicarbonate: to 10g maize fibre containing 1.75%w/w ferulic acid (FA) and 0.1%w/w coumaric acid (CA) was added 100ml 0.5m sodium bicarbonate solution in a 250ml conical flask. The resulting suspension was heated and mixed at 85°C using a hot plate stirrer apparatus. The release of ferulic acid and coumaric acid into solution was monitored over time using hplc. Ferulic acid and coumaric acid yields were as follows:

60 min., FA 20mg, CA 1.6mg; 245 min., FA 100mg, CA 4.3mg; 315 min, FA 127mg, CA 5.6mg; 365 min., FA 126mg, CA 6.2mg. Yields at 365 min are equivalent to 72% and 62% release of available ferulic acid and coumaric acid respectively.

The maize suspension was coarse filtered by pressing through a mesh bag, the recovered solids washed with 10ml deionised water and the filtrates combined prior to centrifugation (4,000 x g, 15 min). The pH of the

- 14 -

supernatant was adjusted to pH 2.5 with concentrated hydrochloric acid followed by extraction with ethyl acetate (3x100ml). Evaporation of the combined ethyl acetate layers to dryness yielded 216mg of yellow/orange solid material comprising 41% ferulic acid and 2.7% coumaric acid.

In the following examples (c) and (d), the production of ferulic acid and coumaric acid from maize fibre was effected by a two step process. Firstly, acid hydrolysis of the fibre was achieved by heating with citric acid solutions, supplied as either a defined quantity of citric acid dissolved in water or as the juice from freshly squeezed lemon or lime fruit. Secondly, the hydrolysis of solubilised cinnamate sugar esters was achieved by the addition of a hydrolytic enzyme preparation yielding ferulic and coumaric acids.

(c) Maize fibre (20g) was mixed with 100ml of citric acid solution (2%) in 250ml conical flask and heated at 126°C for 1 hour. The pH of the maize suspension was raised to pH 5.0 by the addition of 10M sodium hydroxide solution with vigorous mixing. An enzyme preparation (40mg, Hemicellulase, Amano) was added to the suspension and the whole incubated at 50°C for 46.5 hours with mixing at 200rpm. The release of ferulic acid was monitored by hplc as described above. After 1.5 hours incubation a total of 51mg ferulic acid was present in solution, after 46.5 hours this amount had risen to 220 mg.

(d) Maize fibre (200g) was mixed with 1 litre citric acid solution (2%) in a 2L conical flask and heated at 126°C for 1 hour. The maize suspension was separated into insoluble solids and a liquor fraction using a wine press. First

- 15 -

pressing yielded approximately 800ml liquor; the recovered solids were washed with a further 200ml of water and pressed again to give approximately 1 litre of combined liquor fractions. The pH of the maize liquor was raised to pH 7.0 by the addition of 10M sodium hydroxide with vigorous mixing. An enzyme preparation (Biofeed Plus L, 2ml, Novo Nordisk) was added to the liquor and the whole incubated at 60°C for 7.5 hours with mixing at 160 rpm. The release of ferulic acid and coumaric acid was monitored by hplc as described above. After 7.5 hour incubation 1.6g L⁻¹ ferulic acid and 0.1 gL⁻¹ p-coumaric acid were detected in solution. Both cinnamic acids were recovered from aqueous solution by extraction into ethyl acetate as described below, followed by either, base extraction of the solvent to yield the cinnamate sodium salt, or evaporation of the solvent to dryness to yield the cinnamate free acid.

Maize liquor (1L) was brought to pH 3 by the addition of concentrated hydrochloric acid and filtered through diatomaceous earth to remove insoluble material. The filtrate was extracted twice with 300 ml of ethyl acetate and the solvent extracts combined. Evaporation of solvent to dryness yielded 2.6g of orange solid comprising 1.39g ferulic acid and 0.1g coumaric acid, 53.5% and 3.8% purity respectively. In order to recover the cinnamates as their sodium salts, the ethyl acetate was continuously pumped over 10M sodium hydroxide solution (10ml) combined with vigorous mixing of the aqueous phase. This was continued until all but trace quantities of cinnamate had been recovered into the

- 16 -

aqueous phase (approx. 2hrs). The aqueous phase was dried under vacuum at 45°C to yield 11.5 g of cream solid material comprising 1.3g ferulic acid and 0.1g coumaric acid, 11.3% and 0.87% purity respectively.

5 e) Conversion of citric acid treated maize fibre suspension to a digested pulp containing ferulic acid and vanillic acid

4g maize fibre containing ca 1.75% w/w ferulic acid and 20 ml 2% w/v citric acid solution were added to each of three 50ml conical flasks and the whole autoclaved at 126°C for 55 minutes. After cooling the pH of the treated suspension was adjusted to pH 6.0 using 10M sodium hydroxide solution. Flasks were inoculated with spores of either Zyl 714 (IMI 379895) *Aspergillus flavus*, Zyl 759 (IMI 379904) *Aspergillus niger* or *P.chrysogenum* Zyl 860 (IMI 379900) and incubated at 30°C, shaking at 250 rpm on an orbital mixer. Flasks were assayed by hplc for the hydrolytic product ferulic acid, and for the ferulic acid side chain cleavage product vanillin acid.

After 89 hours incubation the concentrations of ferulic acid and vanillic acid in solution for the *P.chrysogenum* experiment were ferulic acid, undetectable; and vanillic acid, 1.35g/L.

Concentrations of ferulic acid and vanillic acid in solution for *Aspergillus flavus*, *Aspergillus niger* respectively after 5 days incubation were 1g/L ferulic acid and 0.3g/l vanillic acid; undetectable ferulic acid and 1.2g/L vanillic acid.

- 17 -

All three fungi reduced the maize fibre suspension to a digested pulp and the fibre fragments to fine solids.

f) Pilot scale processing of maize fibre for release of ferulic acid

5 3 batches of 10kg maize fibre were each suspended in 50L of 2% w/v aqueous citric acid solution. Batch 1 and 2 were heated to 126° for 1 hour and then rapid cooled to 80°C using an Agemore swept surface heating/mixing vessel. The solids were then separated from the liquor using a Vigo 72L winepress, and the solids washed with water to restore the starting volume.

Batch 1 and 2 combined generated 100L of liquor. A sample of this liquor was treated by base hydrolysis to release the available ferulic acid and analysed by hplc , the ferulic acid concentration was 1.5g⁻¹L.

20 The third batch was heated to 134°C for 15 minutes, rapid cooled to 80°C, and pressed using the same apparatus as batches 1 and 2, however the solids were not washed with water after the first pressing. This batch generated 44.5L of liquor. A sample of this liquor was treated by base hydrolysis to release the available ferulic acid and analysed by hplc, the ferulic acid concentration was 1.12g⁻¹L.

25 All three batches of liquor were then combined to give 145L and the concentration of suspended solids measured as 10.5% w/v by refractometry, the pH was measured as pH 3.4. The liquor was then concentrated using a Junior rising/falling plate evaporator at 60°C. 19.5 L of liquor was obtained, the suspended solids level was determined, by

- 18 -

refractometry, to be 50%. A sample of this liquor was treated by base hydrolysis to release the available ferulic acid and analysed by hplc, the ferulic acid concentration was 8.0gL^{-1} .

5 The concentrated liquor was then transferred to a 70L Biolafitte fermenter and diluted with water to give a total volume of 33L. The pH of the liquor was adjusted from pH 3.0 to pH 6.0 with 2.5L 32% w/v sodium hydroxide solution. The liquor was then heated to 45°C . An enzyme, hemicellulase (Amano), was added at a concentration of 0.8gL^{-1} , and the
10 mixture incubated with stirring for 30 hours. After the enzyme treatment, the liquor was analysed by hplc, the ferulic acid concentration was 3.5gL^{-1} .

The pH of the liquor was lowered to pH 3.0 with 82.5%
15 w/v phosphoric acid and the suspended solids removed by centrifugation using a Carr Powerfuge. The ferulic acid was then extracted from the liquor using 50L of butyl acetate (in two batches of 25L). The butyl acetate was overlaid on the liquor and mixed gently in the fermenter, until the
20 concentration of ferulic acid in the butyl acetate layer stopped increasing. 48L of butyl acetate were recovered once the extraction was complete. Analysis of the butyl acetate by hplc gave the ferulic acid concentration as 2.64gL^{-1} .

g) Pilot scale processing of sugar beet for release of
25 ferulic acid

40kg Fibrex sugar beet fibre was suspended in 200L water overnight. This suspension was then heated to 134°C for 30 minutes and then rapid cooled to 80°C (cooling time was 10

- 19 -

minutes) using an Agemore swept surface heating/mixing vessel. The solids were then separated from the liquor using a Vigo 72L winepress, and the solids washed with water to restore the starting volume.

5 200L of liquor was obtained, the pH was measured as pH 4.0, the suspended solids level was determined, by refractometry, to be 9.5% w/v. A sample of this liquor was treated by base hydrolysis to release the available ferulic acid and analysed by hplc, the ferulic acid concentration was 1.03gl⁻¹.

10 The liquor obtained was then concentrated using a Junior rising/falling plate evaporator at 60°C. 31L of liquor was obtained, the suspended solids level was determined, by refractometry, to be 50% w/v. A sample of this liquor was treated by base hydrolysis to release the available ferulic and analysed by hplc, the ferulic acid concentration was 5.2gL⁻¹.

15 The liquor was then transferred to a 70L Biolafitte fermenter and heated to 60°. The pH was adjusted to, and controlled at, pH 6.5 with 20% w/v phosphoric acid. An enzyme, Pentopan BG 500 (Novo Nordisk), was added at a concentration of 1.8gL⁻¹, and the mixture incubated, with stirring, for 24 hours. After the enzyme treatment, the liquor was analysed by hplc, the ferulic acid concentration was 2.55gL⁻¹.

20 The pH of the liquor was lowered to pH 3.0 with 82.5% w/v phosphoric acid and the suspended solids removed by centrifugation using a Carr Powerfuge. The ferulic acid was

- 20 -

then extracted from the liquor using 35L of butyl acetate (in three batches, 2 x 15L, 1 x 5L). The butyl acetate was overlaid on the liquor and mixed gently in the fermenter, until the concentration of ferulic acid in the butyl acetate layer stopped increasing. 32L of butyl acetate were recovered once the extraction was complete. Analysis of the butyl acetate by hplc gave the ferulic acid concentration as 2.10gL⁻¹.

The ferulic acid was then extracted from the butyl acetate by base extraction. The butyl acetate was pumped through 50ml of deionised water in a 500ml Duran bottle. The water was maintained at pH 9.0 using a Metrohm pH Stat titrating 10M sodium hydroxide and mixed using a magnetic stirrer plate and stirrer bar. Once it had passed through the aqueous phase, the butyl acetate was pumped back into the bulk solvent volume. This arrangement extracts the ferulic acid from the solvent phase and concentrates it in the pH 9.0 aqueous phase. The aqueous phase eventually became saturated with ferulic acid and a precipitate was formed which was recovered by filtration under vacuum through Whatman no.41 filter papers. These solids were dried overnight at 50°C in a drying oven. The first batch of solids obtained by this method weighed 34g and contained 24g ferulic acid by hplc analysis. The second batch of solids weighed 18g and contained 8g ferulic acid by hplc analysis.

h) Pilot scale processing of maize fibre for release of ferulic acid by base hydrolysis

- 21 -

1 tonne of maize fibre was suspended in 10,000L of water containing 600 Kg 30.5% sodium hydroxide. This was then heated to 50°C in a 15m³ stainless steel jacketed vessel and mixed for 8 hours. The pH of this suspension was measured as pH 11.8.

The bulk solids were then removed using a scroll decanter, and the liquor obtained transferred to a storage vessel. The pH of the liquor was then adjusted to pH 6.0 with 160 Kg 75% w/v phosphoric acid.

The liquor was then concentrated using an APV evaporator, which gave a final product of 2.5 tonnes. After concentration, the pH of the liquor was lowered to pH 3.0 with 180 Kg 75% w/v phosphoric acid. A sample of this material was removed and analysed for ferulic acid by hplc, the ferulic acid concentration was 2.1gKg⁻¹.

i) Repeat use of citric acid solutions for extraction of ferulic acid from maize fibre

20g of Citric acid was dissolved in 1 litre of RO water, pH was measured as pH 2.20. 200g of maize fibre (supplied by Staley) was added to the citric acid solution and then autoclaved at 121°C for 60 minutes. The solids were then separated from the liquor using a Vigo bench wine press whilst the suspension was still at 80°C. 800ml of liquor was recovered from the initial pressing. The retained solids were then washed with 200 ml RO water, and pressed a second time. 220 ml of liquor was recovered from the second pressing. The liquor obtained from both pressings were added

- 22 -

together to give a total volume of 1.02 L. The pH of the liquor was measured as pH 2.32.

A sample of liquor was treated by base hydrolysis to release the available ferulic acid and analysed by hplc, the
5 ferulic acid concentration was 2.49gL^{-1} . This represents a yield of 1.24% w/w ferulic acid from maize fibre.

The pH of the liquor was returned to pH 2.20 using 2.37g of citric acid. A fresh 200g of maize fibre (supplied by Staley) was added to the liquor and then autoclaved at 121°C for 60 minutes. The suspension was treated as described above, 730ml was recovered from the first pressing, the solids were washed with 270ml of RO water and pressed again, 320ml was recovered from the second pressing. The liquors obtained from both pressings were added together to give a total volume of 1.05L. The pH of the liquor was measured as pH 2.58.

A sample of liquor was treated by base hydrolysis to release the available ferulic acid and analysed by hplc, the
15 ferulic acid concentration was 3.13gL^{-1} . This represents a yield of 0.82% w/w ferulic acid from maize fibre.

Example 1B: biotransformation of product mixtures

A seed stage culture of Rhodotorula glutinis (Zyl 717) was grown for 24 hours at 30°C with shaking at 200 rpm in a 250 mL shake flask containing 50 mL of minimal medium
25 (containing $2\text{g/l KH}_2\text{PO}_4$; 0.2g/l NaCl ; 0.22g/l MgSO_4 ; 0.015g/l CaCl_2 ; 1ml/l trace elements solution; 10ml/l vitamins solution; 4g/l yeast extract; 4g/l glucose). This culture was used to inoculate (2%) a 250 mL shake flask containing 50

- 23 -

mL of the same medium to which was added 300mg of the maize extract produced in example (a) above. This gave an equivalent to 2g L⁻¹ ferulic acid and 0.18g L⁻¹ coumaric acid substrates. The mixture was agitated at 500 rpm at 30° with a dissolved oxygen level of 60% of saturation. Substrate and product concentrations were measured by hplc as the following: 16 hours, ferulic acid 0.68g L⁻¹, vanillic acid 1g L⁻¹, coumaric acid 0.08g L⁻¹, 4-hydroxybenzoic acid 0.05g L⁻¹, 3,4-dihydroxybenzoic acid 0.02g L⁻¹; 18 hours, ferulic acid 0.14g L⁻¹, vanillic acid 1.54g L⁻¹, coumaric acid 0.035g L⁻¹, 4-hydroxybenzoic acid 0.08g L⁻¹, 3,4-dihydroxybenzoic acid 0.05g L⁻¹.

The product mixture can undergo further biotransformation, e.g. with reduction of -CO₂H groups to -CH₂OH and/or -CHO e.g. using Zygorhynchus moelleri or Micromucor isabellinus (see below). Thus the vanillic acid may be converted into a mixture of vanillin and vanillyl alcohol, in variable proportions. Likewise the 4-hydroxybenzoic acid may be converted into a mixture of 4-hydroxybenzaldehyde and 4-hydroxybenzyl alcohol. The minor acid components may undergo corresponding reductions. Thus the end product is a complex mixture of odoriferous compounds, principally of vanilla type. The proportion of aldehydes (such as vanillin) can be increased by biotransformation of the alcohols in the mixture, e.g. using Brevundimonas vesicularis.

Example 1C - Production of vanillic acid from Maize Fibre

- 24 -

Maize fibre (1kg) was subjected to citric acid hydrolysis (5L, 2% solution) followed by pressing and washing of the maize solids to give approximately 5 litres of maize liquor as described in (c) above. The pH of the liquor was adjusted to pH5.8 using 10M sodium hydroxide. A 2L aliquot of the above liquor was transferred into a 5L working volume fermenter and heated to 100°C for 1 minute. The remaining liquor was also heat-treated and stored separately. The fermenter was inoculated with 200 ml of a culture of *Penicillium chrysogenum* (Zyl 860) grown on pH5.8 maize liquor for 30 hours in a 1 litre conical flask (28°C shaking at 250 rpm). The fermenter contents were grown for 15 hours at 28°C while controlling the dissolved oxygen concentration at 30% of saturation. The pH of the culture was not controlled but remained unchanged throughout the incubation period. After 15 hours the volume of the fermenter contents was increased initially to 3.5L and then to 5L after a further 7 hours incubation. Incubation was continued as described previously for a further 3 days after which time the fermenter contents were assayed by hplc as described above. The maize liquor was shown to contain 1.2gl⁻¹ vanillic acid and only a trace quantity of ferulic acid. Fungal biomass was separated from the maize liquor by a single pressing of the fermenter contents through a wine press to give a clarified aqueous product.

Whereas *P.chrysogenum* (Zyl 860) is the currently preferred strain, *Aspergillus flavus* (Zyl 714) and *A.niger* (Zyl 759) are also usable.

- 25 -

Vanillic acid can be recovered from aqueous solution by extraction into ethyl acetate followed by either evaporation of the solvent to dryness or by precipitation of vanillic acid from the solvent by the addition of hexane. For example, a culture broth (900 ml) comprising 567 mg vanillic acid was acidified to pH 3.0 and extracted three times with ethyl acetate (1x400ml, 2x200ml). The solvent extracts were combined, assayed by hplc as described above and found to contain 485mg vanillic acid (86%recovery).

An aliquot (200ml) was taken from the combined ethyl acetate extracts and the volume reduced to approximately 10ml by evaporation. Hexane (40ml) was added slowly to this concentrated extract, accompanied by constant mixing, the precipitated material was recovered by filtration and dried to yield 140mg of pale yellow solid comprising 115mg vanillic acid (82% purity). Recovery from the solvent was 88%, therefore, giving an overall recovery of 76% from the original culture broth.

Example 1D - Production of vanillin from maize fibre

The nutrient content of the clarified liquor from Example 1C was enhanced for growth of *Micromucor isabellinus* by the addition of nutrients as described in Example 4 below. No further vanillic acid was added to the liquor. A culture of *Micromucor isabellinus* (Zyl 849) was grown at 30°C for 3 days on pH 5.8 maize liquor (described in Example 1C) solidified with 1.5% agar. This culture was used to inoculate 20ml of the enhanced clarified liquor described above contained in a

- 26 -

100 ml conical flask. The flask contents were incubated at 30°C with shaking at 250 rpm for 24 hours, prior to being used to inoculate (2.5%) a second identical flask. Incubation conditions were as described previously. The concentrations of vanillic acid and vanillin in solution throughout the process were assayed by hplc as described above. After 24 hours incubation the pH of the liquor was slowly reduced over approximately 1.5 hours from pH5.2 to pH3.7. After 2.25 hours post onset of the pH reduction, 0.02g^L⁻¹ vanillin was detected in solution. After 4.5 hours the vanillin concentration had increased to 0.12g^L⁻¹. At 7.5 hours the vanillin and vanillic acid concentrations were 0.275g^L⁻¹ and 0.81g^L⁻¹ respectively. After 13 hours incubation the vanillic acid concentration had decreased to 0.45g^L⁻¹ and the concentration of vanillin had reached a maximum at 0.35g^L⁻¹.

B) Vanillin from vanillic acid via vanillyl alcohol

'One pot' bioconversions of vanillic acid to vanillin are known, but they generally show low yields and low conversion rates and/or low product concentrations (e.g. EP 453368, FR 2724394). We have found that Zygorhynchus moelleri can be used to produce very high concentrations of vanillyl alcohol (e.g. >5g/l) from vanillic acid. Vanillyl alcohol can be efficiently oxidised to vanillin, e.g. by means of Brevundimonas vesicularis.

Example 2: vanillyl alcohol from vanillic acid

A culture of Zygorhynchus moelleri (Zyl 851) grown on yeast malt agar was used to inoculate a 250mL starter culture flask containing 50mL medium, (20g glucose; 5g (NH₄)₂SO₄; 2g

- 27 -

NaCl; MgSO₄ 0.22g/L; CaCl₂ 0.015g/L, 10mL; trace element solution, 1mL; vitamins solution 10mL; made up to 1 litre with pH6.0 phosphate buffer, 0.2M), containing 2g/L vanillic acid, which was incubated at 30°C with shaking at 200rpm for 24 hours. This starter culture was added to 5 litres of the same medium in a fermenter except that the medium components were dissolved in deionised water and also containing 2g/L vanillic acid. This was stirred at pH 5.2, (30°C) for 24 hours after which the pH was altered to 3.5 over 1 hour, and the temperature kept at 30°C. Dissolved oxygen was maintained at 70% of saturation throughout the process. Assay was by hplc. Prior to the pH being allowed to drop (at the 24 hour stage), the amount of vanillic acid present in the system had not dropped and no products were seen. As the reaction proceeded, various amounts of substrate and nutrients were added as follows: 31.75 hours, 10g vanillic acid and 25g glucose; 47.5 hours, 50g glucose; 55 hours, 20g vanillic acid. Products were measured as being the following: 31.75 hours; vanillic acid, 0.26g/L, vanillyl alcohol, 0.3g/L; 47.5 hours, vanillic acid, 1.17g/L, vanillin, 0.1g/L, vanillyl alcohol, 2.38g/L; 55 hours, vanillic acid, 0.64g/L, vanillin, 0.06g/L, vanillyl alcohol, 3.5g/L; 5 days, vanillic acid, 1.21g/L, vanillin, 0.05g/L, vanillyl alcohol, 6.6g/L; after this stage no further product accumulation was observed. It is also evident that at pH 5.2 no substrate was converted into any products and the bioconversion only started when the pH was allowed to drop to pH 3.5.

Example 3: vanillin from vanillyl alcohol

- 28 -

Sterile nutrient broth (No 2) (50 mL) in a 250 mL shake flask was inoculated with Brevundimonas vesicularis (Zyl 295) and incubated at 30°C with shaking at 200rpm. After 24 hours the addition of 50mg of vanillyl alcohol was followed by the flask being monitored regularly by hplc for both vanillyl alcohol and vanillin. Up to 71 hours post inoculation the amount of vanillyl alcohol decreased to around 10% of the original level and the amount of vanillin increased to approximately 90% molar conversion from substrate.

Identification of the structure of the product was confirmed by nmr spectroscopy.

If the vanillyl alcohol was added at the time of inoculation of the flask then the amount of substrate did not start to decrease until after 16 hours incubation when vanillin was first detected. The overall conversions closely followed those seen when substrate was added after 24 hours.

C) Vanillin from vanillic acid

As mentioned above, the biotransformation of vanillic acid into vanillin is known, but the yields of known methods are poor, making them unattractive in commercial terms. FR-A-2724394 discloses a process, using a basidiomycete, which gives fairly good yields and conversion rates in percentage terms, but whose absolute yields are low. The highest yield of vanillin produced in an example is 628mg/l.

We have found strains capable of affording vanillin at greater than 1g/l and usable in continuous production systems. Preferred microorganisms are strains of Micromucor isabellinus and Aspergillus fumigatus.

- 29 -

Example 4: production of vanillin by M.isabellinus

- (a) A culture of Micromucor isabellinus (Zyl 849) grown on yeast malt agar was used to inoculate a 250 mL starter culture flask containing 50 mL medium (15g glucose; 5g (NH₄)₂SO₄; 2g K₂HPO₄; 0.2g NaCl; 0.2g MgSO₄; 0.015g CaCl₂, trace element solution, 1mL; vitamins solution 10mL; made up to 1 litre with deionised water) containing 2g/L vanillic acid which was incubated at 30°C with shaking at 200rpm for 16.5 hours. This starter culture was added to 5 litres of the same medium in a fermenter with vanillic acid added to a concentration of 1.5g L⁻¹. The fermenter contents were stirred at 30°C with the dissolved oxygen concentration being maintained at 70% of saturation throughout the process. Assay was by hplc as described above. As the reaction proceeded, additional amounts of vanillic acid were added as follows: 22.5 hours, 2.5g; 24 hours, 2.5g; 25.75 hours, 1g; 26.5 hours, 1g; 27.5 hours, 1g; 31 hours, 2.5g. Substrate and product concentrations were measured as being the following: 22.5 hours, vanillic acid 0.6g L⁻¹; vanillin 0.84 g L⁻¹; 24 hours, vanillic acid 1.36g L⁻¹; vanillin 0.96 g L⁻¹; 25.75 hours, vanillic acid 1.15g L⁻¹; vanillin 1.1g L⁻¹; 26.5 hours, vanillic acid 1.27g L⁻¹; vanillin 1.17 g L⁻¹; 27.5 hours, vanillic acid 1.43g L⁻¹; vanillin 1.33 g L⁻¹; 31 hours, vanillic acid 1.16g L⁻¹; vanillin 1.6 g L⁻¹; 46.5 hours, vanillic acid 1.58g L⁻¹; vanillin 1.70 g L⁻¹.

- 30 -

(b) A culture of Micromucor isabellinus (Zyl 849) grown on yeast malt agar was used to inoculate a 500 mL starter culture flask containing 100 mL medium (15g glucose; 5g $(\text{NH}_4)_2\text{SO}_4$; 2g K_2HPO_4 ; 0.2g NaCl; 0.2g MgSO_4 ; 0.015g CaCl_2 , trace element solution, 1mL; vitamins solution 10mL; made up to 1 litre with pH 6.0 phosphate buffer (0.2M)) containing 2g/L vanillic acid which was incubated at 30°C with shaking at 200rpm for 24 hours. This starter culture was added to 5 litres of the same medium in a fermenter with the medium components made up to 1 litre with deionised water and vanillic acid added to a concentration of 1.5g L^{-1} , immediately prior to inoculation. The fermenter contents were stirred at 30°C for 18 hours with the pH of the medium being maintained at pH 5.2 by the addition of 5M sodium hydroxide solution. After this time the medium pH was gradually reduced to 3.7 over 45 minutes by the addition of 5M HCl and temperature was maintained at 30°C. Dissolved oxygen concentration was maintained at 70% of saturation throughout the process. Assay was by hplc as described above. Prior to the pH being allowed to drop at the 18 hour stage, the amount of vanillic acid in the system was measured at 1.48 g L^{-1} ; vanillin was detected in solution at 0.02 g L^{-1} . As the reaction proceeded various amounts of vanillic acid or glucose were added as follows: 19.25 hours, 50g glucose; 20.25 hours, 3g vanillic acid; 21.5 hours, 4g vanillic acid; 23 hours, 4g vanillic acid. Substrate and product concentrations

- 31 -

were measured as being: 19.25 hours, vanillic acid 1.5 g L⁻¹, vanillin 0.1 g L⁻¹; 20.25 hours, vanillic acid 1.16 g L⁻¹, vanillin 0.36 g L⁻¹; 21.5 hours, vanillic acid 1.07 g L⁻¹, vanillin 0.74 g L⁻¹; 23 hours, vanillic acid 1.06 g L⁻¹, vanillin 1.22 g L⁻¹; 26 hours, vanillic acid 0.83 g L⁻¹, vanillin 1.96 g L⁻¹.

Example 5: production of vanillin by A.fumigatus

A spore suspension of Aspergillus fumigatus (Zyl 747) was used to inoculate 5 litres of a minimal medium (ingredients described in Example 4(a), except for the addition of 3g L⁻¹ vanillic acid rather than 1.5g L⁻¹). The fermenter contents were stirred without pH control at 30°C with the dissolved oxygen concentration being maintained at 60% of saturation. Assay was by hplc as described above. Substrate and product concentrations in the culture broth were as follows: 16 hours, vanillin 0.015g L⁻¹; 24 hours, vanillin 0.075g L⁻¹; 40 hours, vanillin 0.69g L⁻¹; 47 hours, vanillin 0.9 lg L⁻¹; 48.5 hours, 0.98g L⁻¹; 53.5 hours 1.02g L⁻¹; 112 hours, vanillic acid 1.32g L⁻¹, vanillin 1.09g L⁻¹

D) Vanillin from ferulic acid

Example 6 Conversion of Ferulic acid to Vanillin by Zyl 581 an NTG derived mutant of Zyl503

Background

ZYL503 converts ferulic acid (FA) to vanillin acid (VA) via the intermediate vanillin. The rate-limiting step in this reaction is FA to vanillin, therefore accumulation of vanillin in culture media is not observed. Based on the NTG

- 32 -

mutation method described below a ZYL503 mutant has been produced that accumulates vanillin in culture media when supplied with FA.

(a) NTG Mutagenesis of ZYL503

5 ZYL503 was grown to mid-exponential phase in 10ml nutrient broth, harvested, washed once in 100mM sodium citrate buffer (pH 5.5), resuspended in 10ml of this buffer containing 0.1g l⁻¹ NTG, and incubated at room temperature. At five minute intervals 0.5ml aliquots were taken, washed twice in 50mM
10 potassium phosphate buffer, and resuspended in 1ml of this buffer. These samples were then diluted in 50mM phosphate buffer and spread onto a minimal medium agar (pH7, 0.4M phosphate buffered), that contained 20g l⁻¹ glucose or 1 g l⁻¹ vanillin plus 2 g l⁻¹ yeast extract and 2 g l⁻¹ ferulic acid.
15 Plates were incubated at 30°C for 24-48h. Resultant colonies unable to grow on vanillin plates were selected. Zyl 581 was obtained based on this procedure.

(b) Conversion of commercial ferulic acid (free acid) to vanillin by Zyl 581

20 A preculture of Zyl 581 was grown in a 250 ml shake flask containing 50ml minimal medium (g l⁻¹:
(NH₄)₂SO₄, 5; K₂HPO₄, 2; NaCl, 0.2; Yeast extract, 0.2, glucose, 20:
10ml of a solution containing 0.1 M MgSO₄/0.01M CaCl₂; 10ml vitamin solution and 1ml trace elements solution at 200rpm,
25 30°C for 24h. This culture was then used to inoculate a bioreactor filled with 1.2 l of the same medium with pH control at 8.0 using 2M NaOH, oxygen control at 70% with a stirrer speed cascade of 100-400rpm air flow 1.3 vvm. Prior

- 33 -

to inoculation 2 g l⁻¹ ferulic acid (Lancaster, 99%) was added as the fermentation proceeded as indicated in the table below. An identical reactor was also set up with pH control at 8.5. Reaction products were quantified by HPLC.

5 pH 8.0

TO340

	Time (h)	FA (g l ⁻¹)	VA (g l ⁻¹)	Vanillin (g l ⁻¹)	FA added (g l ⁻¹)
	0	1.8881	0.000	0.000	
	19.7	0.616	0.298	0.545	
	21.2				1.380
10	21.7	1.953	0.433	0.460	
	23.7	1.865	0.347	0.619	
	25.7	1.806	0.798	0.260	
	27.7	1.786	0.912	0.182	
	43.4	1.105	1.039	0.278	
15	48.0	0.387	1.245	0.577	

pH 8.5

TO341

	Time (h)	FA (g l ⁻¹)	VA (g l ⁻¹)	Vanillin (g l ⁻¹)	FA added (g l ⁻¹)
20	0	1.891	0.000	0.000	
	19.7	0.520	0.000	0.545	
	21.2				1.450
	21.7	1.802	0.000	1.161	
	23.7	1.380	0.000	1.418	
25	25.7	1.074	0.025	1.475	
	26.0				0.970
	27.7	1.829	0.038	1.521	

- 34 -

Time (h)	FA (g l ⁻¹)	VA (g l ⁻¹)	Vanillin (g l ⁻¹)	FA added (g l ⁻¹)
43.4	0.424	0.083	2.247	
44.8				1.540
48.0	1.871	0.115	2.193	

5 At pH8 vanillic acid was the predominant transformation product from ferulic acid while at pH 8.5 vanillin was the predominant product. After 43h at pH 8.5 the vanillin concentration was 2.247 g l⁻¹ a molar yield of 73% for the ferulic acid consumed.

10 (c) Conversion of sugar beet derived ferulic acid (sodium salt) to vanillin by Zyl581

15 Ferulic acid obtained from Pentopan 500BG treated sugar beet liquor was extracted with butyl acetate, then back extracted into base to give the ferulic acid sodium salt (70.8% w/w ferulic acid as free acid).

20 Zyl581 was grown in a bioreactor as described above at pH 8.5 except that prior to inoculation 5.65 g/l sugar beet ferulic acid extract was added (4 g l⁻¹ available ferulic acid present as sodium salt). After 40h the ferulic acid concentration in the reactor was 0.05g/l, vanillin acid 0.21 g/l and vanillin 1.69 g/l. This represents a molar yield for vanillin of 54%. 72% of the ferulic acid added could be accounted for as vanillic acid, vanillin or remaining ferulic acid.

25

(d) Recovery of Vanillin from spent culture broth

- 35 -

The culture broth from the Zyl581 fermentation was taken, cells removed by centrifugation, the broth adjusted to pH 7.5, overlaid with an equal volume of butyl acetate and stirred overnight. The butyl acetate was evaporated under vacuum and the resultant solid washed with 10ml hexane, recovered by filtration and dried at 40°C overnight.

400mg of the extracted solid from the Zyl581 fermentation (95% w/w vanillin by HPLC), was purified using the following procedure. The product was dissolved in 10ml diethyl ether to which 40 mg of silica (70-200 μ m) was added. The ether was evaporated leaving the solid adsorbed to the silica. The silica was washed with 500ml of a 2% (v/v) solution of ethyl acetate in petroleum ether (60-80 fraction). This solution was filtered through a glass sinter and evaporated under vacuum to yield a white amorphous solid containing greater than 99% w/w vanillin (277mg).

E) In situ product removal ("ISPR")

The principle involves carrying out a biotransformation in an aqueous phase which is in contact with, or is contacted with, an immiscible phase into which a product can pass (exclusively or preferentially). Possible advantages include a) the protection of a product from further reaction in the aqueous phase; b) avoidance of inhibition of product formation by a microorganism by high product concentrations; c) enabling equilibrium reactions to convert a larger proportion of starting material into product; and d) ease of

- 36 -

isolation of products. Furthermore ISPR can assist in the development of continuous systems.

ISPR is readily applied to systems where a polar substrate (e.g. a carboxylic acid such as ferulic or vanillic acid) is converted into a less polar product, which will be preferentially extracted by a nonpolar solvent such as a plant oil material, preferably food grade. Polar byproducts will also tend to stay in the aqueous phase.

Examples 7 and 8 correspond to Examples 4 and 5 but make use of ISPR.

Depending on the system, the immiscible phase may contact the aqueous phase while biotransformation is proceeding therein, or it may contact portions of the aqueous phase which have been withdrawn (temporarily) from the bioreactor. The withdrawn phase may be treated (e.g. by adjustment of pH) to facilitate extraction by the immiscible phase.

Example 7: production of vanillin by *M.isabellinus* with ISPR

A 5 litre culture of *Micromucor isabellinus* (Zyl 849) was grown in a fermenter as described in Example 4b. The fermenter contents were stirred at 30°C for 20 hours with the pH of the medium maintained at 5.2 by the addition of 5M sodium hydroxide solution. After this time the medium pH was gradually reduced to 3.8 over 45 minutes by the addition of 5M HCl and was maintained at this pH thereafter; temperature was maintained at 30°C. Dissolved oxygen concentration was maintained at 70% of saturation throughout the process. Assay was by hplc as described above. The progress of the

- 37 -

reaction was monitored until the concentration of vanillin in the culture broth reached 0.96 g L^{-1} . Throughout this period vanillic acid was added to the medium to maintain a concentration of 1.5 g L^{-1} . At this time an external

5 vanillin extraction system was activated as follows: culture medium was continuously pumped from the fermenter through a filtration device such that the biomass was retained within the fermenter; culture medium exiting the fermenter was heated to 60°C and adjusted to pH 6.5 by the addition of 10M sodium hydroxide solution; this medium was fed into an
10 extraction vessel containing 5 litres of sunflower oil; the aqueous phase (maintained at 1 litre volume) and the oil phase were stirred vigorously by the use of an overhead stirrer to effect continuous selective extraction of vanillin from the culture medium into the oil phase; vanillic acid did
15 not extract into the oil and remained entirely in the aqueous phase; this aqueous phase was continually pumped back into the fermenter. Throughout the process, the volume in the fermenter and the volume in the extraction vessel remained
20 relatively constant. The concentration of vanillic acid in the fermenter was maintained at approximately 1.5 g L^{-1} and the concentration of vanillin in the fermenter was maintained at a maximum of 1.5 g

L^{-1} . Using this continuous external extraction facility the
25 5 litre culture of Micromucor isabellinus produced 18.9g vanillin over an operational period of approximately 20 hours. Vanillin can be recovered from sunflower oil by extraction into water, or alcohol, e.g. methanol or ethanol,

or into an alcohol/water mixture. A suitable mixed solvent is 80% ethanol and 20% water. After evaporation of the extraction solvent, vanillin can be further purified by traditional techniques such as recrystallisation, sublimation etc.

Example 8: production of vanillin by *A.fumigatus* with ISPR

A sterile glass column (60mL volume) packed with a stainless steel support was filled with a minimal medium (ingredients described in Example 4) containing 2g L⁻¹ vanillic acid and inoculated with spores of *Aspergillus fumigatus* (Zyl 747). Air was pumped into the base of the column in order to aerate the system and effect efficient mixing. The column was allowed to stand at room temperature (22°C) for 70 hours. After this time substantial growth of the fungus had occurred and all had adhered to the stainless steel support material. The concentration of vanillin in solution was measured as 0.65g L⁻¹. Maintaining aeration as described above, the contents of the column were continuously pumped out of the column into a separate extraction vessel containing 500mL sunflower oil and 200mL of the same minimal medium described previously. In addition, 1g vanillic acid was added to the aqueous phase. The contents of the extraction vessel were mixed thoroughly and the aqueous phase continuously pumped back through the column. Incubation of the column contents at approximately 30°C was achieved by jacketing the column with silicon tubing and pumping water through at a temperature of 34°C. The concentration of vanillin in both the oil and aqueous phase inside the

- 39 -

extraction vessel was assayed (hplc, as described above) at intervals over an eight day period. Results, expressed as total vanillin yield from the system, were as follows: 24 hours, 113mg; 48 hours, 140mg; 72 hours, 269mg; 96 hours, 385mg; 168 hours, 597mg; 192 hours, 566mg.

The suitability of the system of example 8 for long-term continuous use is demonstrated by Example 9.

Example 9: continuous production of vanillin using ISPR

A spore suspension of Aspergillus fumigatus (Zyl 747) was used to inoculate 3 litres of a minimal medium (ingredients described in Example 9 except for the addition of 3.3g L⁻¹ vanillic acid rather than 1.5g) in a fermenter (5L working volume). The fermenter contents were stirred without pH control at 30°C with the dissolved oxygen concentration being maintained at 60% of saturation throughout the process. Assay was by hplc as described above. After 24 hours incubation, 0.15g L⁻¹ vanillin was detected in solution. At this time 2 litres of sunflower oil containing 0.5g L⁻¹ vanillic acid was added to the fermenter and incubation was continued as described previously. Over a period of 28 days, the oil phase in the fermenter was removed at frequent intervals and replaced with fresh oil containing 0.5g L⁻¹ vanillic acid. Vanillin production expressed as the total vanillin yield from the system was as follows:

Time (hours)	Vanillin yield (g)
48	0.64
72	1.43
96	2.71

- 40 -

	168	4.60
	192	5.12
	216	5.58
	240	6.20
5	264	6.87
	336	9.16
	360	10.32
	384	11.50
	408	12.25
10	432	12.80
	504	13.80
	575	14.50
	665	16.26

15 After 665 hours the experiment was terminated; however, the fungus was still actively producing vanillin.

G) Use of *Pseudomonas putida* for vanillic acid production

Example 10 Conversion of ferulic acid to vanillic acid

20 A nutrient agar plate culture of *Pseudomonas putida* (zyl 503) was used a source of inoculum for 50 ml of growth medium (5 g ferulic acid; 20 g glucose; 2g KH_2PO_4 ; 5g $(\text{NH}_4)_2\text{SO}_4$; 0.2g NaCl; 0.22 g MgSO_4 ; 0.015g CaCl_2 ; 1 ml trace elements solution; 10 ml vitamins solution, made up to 1 litre with 0.2 M, pH 7.0 phosphate buffer) in a 250 ml conical shake flask. The
25 culture was incubated at 30°C shaking at 250 rpm and assayed by hplc as described above. As the reaction proceeded,

- 41 -

additional amounts of ferulic acid were added as follows:
24.5 hours 0.25 g; 48 hours 0.125 g; 72 hours 0.05 g; 90
hours

0.25 g; 96 hours 0.5 g. Substrate and product concentrations

5 were measured as being the following: 20.5 hours, ferulic
acid 2.71 gL^{-1} , vanillic acid 2.08 gL^{-1} ; 24.5 hours, ferulic
acid 1.51 gL^{-1} , vanillic acid 3.01 gL^{-1} ; 48 hours, ferulic acid
 2.4 gL^{-1} , vanillic acid 7.0 gL^{-1} ; 72 hours, ferulic acid 2.08
 gL^{-1} , vanillic acid 9.5 gL^{-1} ; 96 hours ferulic acid 4.47 gL^{-1} ,
10 vanillic acid 12.11 g L^{-1} ; 160 hours, ferulic acid 4.45 gL^{-1} ,
vanillic acid 19.05 gL^{-1} .

Example 11 Production of vanillic acid from maize fibre

To 30 g maize fibre in a 250 ml conical flask was added 100
15 ml of 8% w/v citric acid solution. The flask contents were
heated at 85°C with efficient mixing for 16 hours. After
this time, the stirred maize fibre suspension was neutralised
by the dropwise addition of sodium hydroxide solution (10M).
A hydrolytic enzyme preparation ($500 \mu\text{l}$, Biofeed Plus, Novo
20 Nordisk) was added to the suspension and the whole incubated
at 45°C with continued mixing for 24 hours. Analysis was by
hplc as described above. After 24 hours 3.3 gL^{-1} ferulic acid
was detected in solution.

25 A culture of *Pseudomonas putida* (zyl 503) grown on nutrient
agar was used to inoculate a 250 ml shake flask containing 50
ml of minimal salts medium (4 g ferulic acid; 20 g glucose; 5
g $(\text{NH}_4)_2 \text{SO}_4$; 0.2 g NaCl; 2 g K_2HPO_4 ; 0.22 g MgSO_4 ; 0.015 g

- 42 -

CaCl₂; 1 ml trace elements solution; 10 ml vitamins solution made up to 1 litre with pH 7.0 phosphate buffer 0.2 M. The flask contents were incubated at 30°C shaking at 250 rpm for 24 hours. After this time the culture was harvested by centrifugation (4000 x g, 20 minutes), washed once with 0.2 M pH 7.0 phosphate buffer to remove residual non maize derived ferulic or vanillic acid and finally resuspended in 2 ml of the same buffer (x 25 concentration). This concentrated cell suspension was added to a 20 ml aliquot of the hydrolysed maize suspension described above contained in a 100 ml conical flask. The concentration of ferulic acid in solution was measured as 2.8 gL⁻¹ prior to incubation at 30°C with shaking at 250 rpm. The concentration of vanillic acid present throughout the incubation period was measured as being the following: 4 hours, 0.11 gL⁻¹; 24.5 hours, 1.44 gL⁻¹; 29.5 hours, 1.98 gL⁻¹; 31 hours, 2.0 gL⁻¹.

H) Transformations of other substrates

The conversion of ferulic acid into vanillic acid as in example 1 is the conversion of a cinnamic acid (AR-CH=CH-CO₂H) to a benzoic acid (AR-CO₂H). This can be applied to other cinnamic acids, e.g. coumaric acid (4-hydroxycinnamic acid) and caffeic acid (3,4-dihydroxycinnamic acid).

Example 12: 4-hydroxybenzoic acid from coumaric acid

A seed stage culture of Rhodotorula glutinis (Zyl 702) was grown for 24 hours at 30°C with shaking at 200 rpm in a 250 mL shake flask containing 50 mL of a minimal medium (as defined in Example 1). This culture was used to inoculate (2%) a 250 mL shake flask containing 50 mL of the same

- 43 -

minimal medium with the addition of 200 mg coumaric acid to give a final concentration of 4 gL⁻¹. Incubation conditions were as described previously and assay was by hplc as described above. Substrate and product concentrations were measured as the following: 18 hours, coumaric acid 1.75 gL⁻¹, 4-hydroxybenzoic acid 1.28g L⁻¹, 3,4 dihydroxybenzoic acid 0.27 gL⁻¹; 22 hours, coumaric acid 0.36 gL⁻¹, 4-hydroxybenzoic acid 1.92 gL⁻¹, 3,4-dihydroxybenzoic acid 0.44 gL⁻¹; 23 hours, coumaric acid 0.12 gL⁻¹, 4-hydroxybenzoic acid 2.10 gL⁻¹, 3,4-dihydroxybenzoic acid 0.47 gL⁻¹.

The conversion of vanillic acid to vanillin as in example 4 is the conversion of a hydroxybenzoic acid to a hydroxybenzaldehyde. This can be applied to other benzoic acids, particularly hydroxybenzoic acids, e.g. 4-hydroxybenzoic acid (as produced in Example 12) or 3,4-dihydroxybenzoic acid (protocatechuic acid).

Example 13: conversion of 4-hydroxybenzoic acid to 4-hydroxybenzaldehyde and 4-hydroxybenzyl alcohol

A culture of Zygorhynchus moelleri (Zyl 851), grown on yeast malt agar, was used to inoculate a 250 mL conical flask containing 42 mL of culture medium containing 100 mg 4-hydroxybenzoic acid. The culture broth was incubated at 30°C with shaking at 200 rpm. The progress of the reaction was assayed by hplc as described above. Substrate and product concentrations were measured as being the following: 24 hours, 4-hydroxybenzoic acid 2.26g L⁻¹, 4-hydroxybenzaldehyde trace amount, 4-hydroxybenzyl alcohol trace amount; 42 hours, 4-hydroxybenzoic acid 1.06g L⁻¹, 4-hydroxybenzaldehyde 0.53g

- 44 -

L⁻¹, 4-hydroxybenzyl alcohol 0.55g L⁻¹; 66 hours, 4-hydroxybenzoic acid 0.1 g L⁻¹, 4-hydroxybenzaldehyde trace amount, 4-hydroxybenzyl alcohol 2.6g L⁻¹.

Example 14: conversion of 4-hydroxybenzoic acid to 4-hydroxybenzaldehyde

A culture of Trichoderma koningii (Zyl 751) grown on yeast malt agar, was used to inoculate 50 mL of a minimal medium (as defined in Example 4a) in a 250 mL conical flask. Prior to inoculation, 150mg (3 gL⁻¹) 4-hydroxybenzoic acid was added to the medium followed by incubation of the whole at 30°C with shaking at 200 rpm. Assay was by hplc and tlc as described above. After approximately 30 hours incubation, hplc analysis detected 0.3 gL⁻¹ 4-hydroxybenzaldehyde in solution. This observation was further supported by tlc analysis which revealed the presence of material at R_f 0.59 consistent with a reference sample of 4-hydroxybenzaldehyde. The observed product also gave a positive colour reaction with dinitrophenylhydrazine solution.

Example 15: conversion of 3,4-dihydroxybenzoic acid to 3,4-dihydroxybenzaldehyde

A culture of Zygorhynchus moelleri (Zyl 851), grown on yeast malt agar, was used to inoculate a 250 mL conical flask containing 50 mL of culture medium containing 100mg vanillic acid. The culture broth was incubated at 30°C with shaking at 200 rpm. The progress of the reaction was assayed by hplc as described above. After 42 hours incubation, approximately 50% of the vanillic acid had been converted to vanillyl alcohol. At this time, 100mg of 3,4-dihydroxybenzoic acid

- 45 -

(e.g. from Example 12, or extracted from onion skins) was added to the culture and incubation continued. After a further 6 hours incubation a new product corresponding to 3,4-dihydroxybenzaldehyde was detected at a concentration of approximately 0.20g L⁻¹. After 24 hours this product concentration had increased slightly to approximately 0.025g L⁻¹. After 48 hours the 3,4-dihydroxybenzaldehyde had been lost from the solution.

Example 16; Conversion of benzoic acid to benzaldehyde

In a 1 litre flask, 200 mL of medium, (50g glucose; 5g (NH₄)₂SO₄); 2g K₂HPO₄; 0.2g NaCl; 0.22g MgSO₄; 0.015g CaCl₂ 1ml trace element solution, 1mL; 10ml vitamins solution; made up to 1 litre with deionised water), was inoculated with a 10μL loopful of spores of Trichoderma koningii (Zyl 751) after the addition of 50mg of benzoic acid. This was incubated at 30°C with shaking at 200rpm. By hplc analysis, benzaldehyde was first detected in the culture broth at 25 hours incubation. Over the next 5 hours benzaldehyde concentration rose to 0.165g/L; at this time a concentration of 0.1g benzyl alcohol was also present in the solution.

The conversion of vanillyl alcohol into the aldehyde in example 3 can also be applied to other benzyl alcohols, e.g. 4-hydroxybenzyl alcohol as produced in example 14.

Example 17: conversion of 4-hydroxybenzyl alcohol to 4-hydroxybenzaldehyde

The methodology described in Example 17 was followed but at the 24 hour stage of growth of the organism 4-hydroxybenzyl alcohol was added to the flask to give a final

- 46 -

concentration of 1 mg/mL. Monitoring the progress of the reaction by hplc showed that the amount of substrate dropped to 10% of the original after a further 28 hours at which stage 4-hydroxybenzaldehyde had reached a concentration of 0.37 mg/mL.

NB the yields of the aldehydes in examples 14 - 17 could doubtless be improved by the use of ISPR.

I) **Selective screening for organisms producing aldehydes from carboxylic acids**

i) Preparation of agar plates

Minimal salts agar: 20g glucose; 5g $(\text{NH}_4)_2\text{SO}_4$; 2g K_2HPO_4 ; 0.2g NaCl; 0.22g MgSO_4 ; 0.015g CaCl_2 ; 10ml trace element solution; 1ml vitamins solution; 20g agar and 2g vanillic or ferulic acid made up to 1 litre with either deionised water (fungal isolation) or with 0.2M, pH 7.0, sodium phosphate buffer (bacterial isolation).

Filter paper (Whatman No.1) was cut into discs with a diameter of 90 mm and sterilised by autoclaving. Single discs were placed into 90mm sterile petri dishes prior to the pouring of a minimal salts agar described above.

ii) Preparation of soil samples

To 2ml deionised water was added approximately 100mg soil.

The resulting suspension was mixed thoroughly (vortex mixer); allowed to stand at room temperature (22°C) for 1 hour followed by further mixing to distribute suspended material. The macroscopic solids were allowed to settle for

- 47 -

approximately 10 minutes and the supernatant (100 μ l) applied to the prepared minimal salts agar plates using a spread plate technique. Plates were incubated at 28°C until colony development was observed (approx. 5 days)

5

iii) Selective visualisation of aldehyde producing strains

In order to visualise vanillic acid or ferulic acid biotransformation products the following procedure was followed:

Agar was lifted from the base of each petri dish by inserting a spatula beneath the filter paper disc, followed by the injection of 1ml of dinitrophenyl hydrazine (DNP) solution (0.4% DNP in 2M HCl). The agar was replaced in the dish and the DNP solution allowed to permeate through the agar. Colonies producing aldehyde products were visualised by the presence of an orange/red zone surrounding the colony against a pale yellow background. Colonies producing alcohol products were visualised by the presence of a dark yellow zone surrounding the colony, against the pale yellow background.